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PERMEABILITY OF AMINO ACIDS INTO LIPOSOMES

MAKOTO NAOI, MISAKO NAOI, TOMIKO SHIMIZU, A.N. MALVIYA * and KUNIO YAGI

Institute of Biochemistry, Faculty of Medicine, University of Nagoya, Nagoya 466 (Japan)

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Summary

1. A simple and rapid assay for the measurement of permeability of amino acids into liposome membrane was carried out by using the liposomes trapping D-amino acid oxidase (D-amino acid: O_2 oxidoreductase (deaminating), EC 1.4.3.3) inside the membrane.

2. Permeability of amino acids into liposomes depended on the lipid composition of the membrane. Permeability of amino acids into phosphatidylcholine-cholesterol liposomes depended critically on temperature.

3. Permeability also depended on the structure of amino acids. The order of permeability was norvaline > isoleucine > leucine > phenylalanine > tryptophan > methionine > tyrosine, valine > threonine > serine > alanine > glycine.

Introduction

Since Bangham, Standish and Watkins [1] proposed their sample of lipid dispersion as a membrane model, it has been demonstrated that various marker compounds could be trapped into the aqueous regions inside the lipid bilayer of an intact membrane separating the compartments from the external medium. Incorporation of proteins into liposomes has been reported [2,3]. Gregoriadis, Leathwood and Ryman [4] reported the entrapment of *Aspergillus niger* amyloglucosidase (EC 3.2.1.3) into liposomes, and Kataoka, Williamson and Kinsky [5] described the preparation of liposomes trapping hexokinases, glucose-6-phosphate dehydrogenase and β-galactosidase as macromolecular markers.

We tried to trap D-amino acid oxidase into the liposomes and to determine D-amino acids incorporated across the membrane. The present paper describes this assay for the determination of permeability of different amino acids and the composition of the membrane which affects the permeability.

* Visiting professor from Department of Biochemistry, S.N. Medical College, Agra, India.

Materials and Methods

Materials

D-Amino acid oxidase was prepared from hog kidney by the method of Yagi et al. [6]. Phosphatidylcholine was prepared from egg yolks by the method of Singleton et al. [7]. Bovine phosphatidylethanolamine, phosphatidylserine, dipalmitoyl phosphatidylcholine, egg lysolecithin, trilinolein, tristearin and phosphatidic acid were obtained from P-L Biochemicals Inc., Milwaukee. Stearoylamine and D-amino acids were purchased from Nakarai Chemicals Co., Kyoto, and dicetyl phosphate from Pfaltz and Bauer, Inc., Stanford. Cholesterol was purchased from Sigma Co., St. Louis.

Preparation of liposomes

Liposomes were prepared from phosphatidylcholine alone or from a mixture of phosphatidylcholine and cholesterol and/or other phospholipids. Phosphatidylcholine (50 μmol), dissolved in chloroform, was mixed with other lipids dissolved in the same solvent, dried in vacuo on a rotary evaporator to form a film, and was further dried in a desiccator over KOH pellets for 2 h. To this film, was added 1 μmol of D-amino acid oxidase dissolved in 5 ml of 10 mM Tris · HCl buffer (pH 8.5) containing 50 mM NaCl, and the mixture was stirred at 5° C for 15 min under argon atmosphere. While being stirred, lamellar structure was formed. In some experiments, the suspension was sonicated at 5° C for 60 min under a stream of argon gas by using a Sonifier B-12 (power level 2). The solution was left overnight at 5° C, and was centrifuged in a Beckman Spinco L_5-50 centrifuge at 100 000 $\times g$ for 60 min. The pellet was resuspended in 8 ml of the buffer and centrifuged as described above. The washing procedure was repeated twice more. Then the pellet obtained was suspended in 2 ml Tris · HCl/NaCl buffer (pH 8.5) and kept at 5° C.

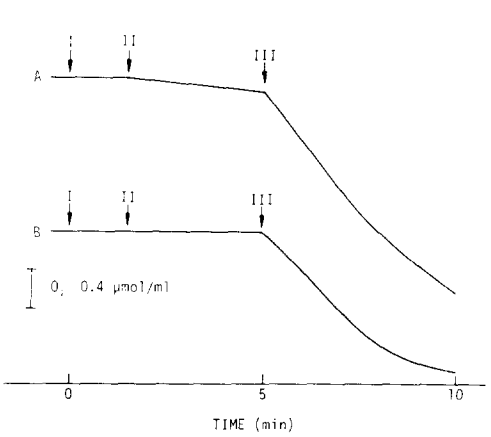
Assay for enzyme activity

Enzyme activity was measured in a way similar to the previously reported one [6]. Oxygen uptake in a closed vessel was recorded polarographically by using a Bioxygraph (Kyusui Kagaku Kenkyusho Co., Tokyo).

Phosphatidylcholine concentration was calculated from the content of inorganic phosphate [8] or from the weight of a dried sample. Protein concentration was measured by the method of Lowry et al. [9].

Results and Discussion

Upon addition of D-alanine to the phosphatidylcholine liposomes prepared to trap D-amino acid oxidase, a slow oxygen consumption was observed (Fig. 1A). The rate of oxygen consumption did not change with time. It was observed that preincubation of liposomes in 0.1 M sodium pyrophosphate buffer (pH 8.3) did not increase the oxygen uptake. This was also the case for the preincubation with more hypotonic solution (1 mM sodium phosphate buffer, pH 8.3). The following addition of Triton remarkably increased the oxygen uptake. All these results indicate that the enzyme was trapped into the inside of the liposomes and that the oxygen uptake can be ascribed to the



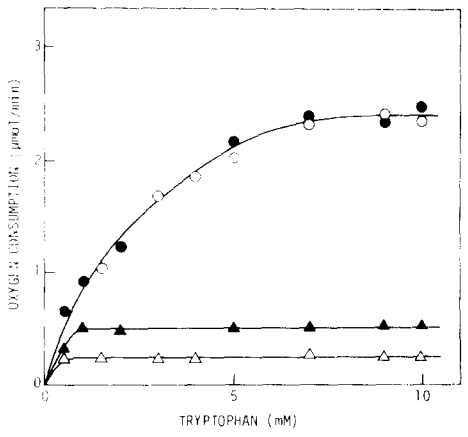


Fig. 1. Oxidation of D-alanine by liposomes trapping D-amino acid oxidase. To 2.5 ml of 0.1 M sodium pyrophosphate buffer (pH 8.3), were added the suspension of liposomes (0.1 ml containing 1 nmol of the enzyme) at I and 0.3 ml of 1 M D-alanine at II. At III, Triton X-100 (0.03 ml of 10% solution) was added. Curve A, for liposomes prepared from phosphatidylcholine; curve II, for liposomes composed of equimolar amounts of phosphatidylcholine and cholesterol. The reaction temperature was 30°C.

Fig. 2. Effect of the concentration of substrate on its oxidation by the liposomes trapping D-amino acid oxidase. To the suspension of phosphatidylcholine liposomes (2.9 ml, enzyme 3.2 nmol) in 0.1 M sodium pyrophosphate buffer (pH 8.3), 0.03 ml of tryptophan was added. Tryptophan concentration was expressed in terms of D-form. The concentration of Triton X-100 was 0.1% in final. The reaction temperature was 30°C. Open circles, DL-tryptophan in the presence of Triton; filled circles, D-tryptophan in the presence of Triton; open triangles, DL-tryptophan in the absence of Triton; filled triangles, D-tryptophan in the absence of Triton.

oxidation of the substrate penetrated into the liposomes, but not to that of the substrate catalyzed by the leaked enzyme.

In the case of liposomes prepared from an equimolar mixture of phosphatidylcholine and cholesterol, oxygen uptake was not observed until the addition of the detergent, excluding further the possibility of the oxidation of the substrate by free enzyme (Fig. 1B).

The trapping of the enzyme into liposomes was found to be affected by the lipid composition. The data are summarized in Table I. In phosphatidylcholine liposomes, approximately 1.3% of the total amount of the enzyme used for the preparation was trapped. The presence of cholesterol in the membrane increased the amount of the trapped oxidase, in proportion to the increase in molar ratio of cholesterol to phosphatidylcholine, in the range of cholesterol concentrations examined. The coexistence of stearoylamine with phosphatidylcholine markedly elevated the trapping of the enzyme.

Relation between the permeability and the oxidation rate

To check the validity of adopting the above preparation for the measurement of permeability of liposomal membrane, the experiment shown in Fig. 2 was performed. When the liposomes were treated by Triton, the maximum oxidation rate of D-tryptophan by the preparation of the liposomes trapping D-amino acid oxidase was 1030 and that of the free enzyme was 1000 in terms of molecular activity. The K_m value obtained by the trapped enzyme was 2.1 mM, which was similar to that of the free enzyme (2 mM). When DL-tryptophan, the

TABLE I

TRAPPING OF D-AMINO ACID OXIDASE INTO LIPOSOMES PREPARED FROM PHOSPHATIDYLCHOLINE AND OTHER LIPIDS

Lipid composition	Molar ratio	Rate of trapping * (%)
Phosphatidylcholine		1.3
Phosphatidylcholine-cholesterol	1 : 0.2	1.7
Phosphatidylcholine-cholesterol	1 : 0.5	2.3
Phosphatidylcholine-cholesterol	1 : 1	3.5
Phosphatidylcholine-cholesterol	1 : 2	11.0
Phosphatidylcholine-phosphatidylethanolamine	1 : 1	5.6
Phosphatidylcholine-phosphatidylserine	1 : 1	1.1
Phosphatidylcholine-dipalmitoyl phosphatidylcholine	1 : 1	1.2
Phosphatidylcholine-lysolecithin	1 : 1	0.3
Phosphatidylcholine-phosphatidic acid	1 : 0.2	2.2
Phosphatidylcholine-dicetyl phosphate	1 : 1	5.4
Phosphatidylcholine-stearoylamine	1 : 0.2	22.0
Phosphatidylcholine-cholesterol-stearoylamine	0.7 : 0.2 : 0.1	20.1
Phosphatidylcholine-trilinolein	1 : 1	1.0
Phosphatidylcholine-tristearin	1 : 1	1.0

* The rate of trapping of the enzyme was calculated as percent of the trapped enzyme to the total amounts of the enzyme used for the preparation of liposomes.

concentration of which was identical to the above in terms of D-form, was used, the same oxygen uptake was observed. On the other hand, in the case of intact liposomes, D-tryptophan was oxidized at the same rate when the substrate concentration was raised more than 1 mM. Upon addition of DL-tryptophan, the concentration of which was identical to the above in terms of D-form, the rate was reduced exactly to the half of that of D-tryptophan. These results indicate that in the case of intact liposomes, the membrane permeability is the rate-determining factor for the oxidation of D-tryptophan, if the substrate concentration is raised more than 1 mM, and that both D- and L-forms of tryptophan equally penetrate into the liposomes at the same rate. These phenomena were also the cases for other amino acids.

Effect of lipid composition of the membrane on the permeability of amino acids

The relation between lipid composition of the membrane and permeability of amino acids was examined. The rate of oxidation of D-alanine by intact liposomes was expressed as percent of that by liposomes in the presence of Triton X-100. As shown in Table II, the permeability largely changed due to the lipid composition of liposomes. Temperature of the reaction mixture also affected the incorporation of amino acids. However, the temperature profile was different due to the different lipid compositions of liposomes. In the case of phosphatidylcholine-cholesterol liposomes, raising of temperature accelerated the incorporation and further raising reduced the incorporation. Upon mixing of lysolecithin with phosphatidylcholine, the permeability of amino acids into the liposomes increased remarkably but the dependence on temperature was not observed. On the other hand, the mixing of dipalmitoyl phosphatidylcholine with phosphatidylcholine caused no incorporation of D-alanine at various tem-

TABLE II

EFFECT OF LIPID COMPOSITION ON LIPOSOMES OF THE OXIDATION OF D-ALANINE

Lipid composition	Molar ratio	Rate of oxidation *					
		Temperature					
		15°C	20°C	25°C	30°C	35°C	40°C
Phosphatidylcholine		0	0	2.5	7.0	7.5	7.0
Phosphatidylcholine-cholesterol	1 : 0.1	2.5	8.7	12.5	14.5	14.3	13.8
Phosphatidylcholine-cholesterol	1 : 0.25	2.5	7.0	11.5	14.5	14.3	13.8
Phosphatidylcholine-cholesterol	1 : 0.5	1.3	4.5	7.0	7.5	7.5	7.5
Phosphatidylcholine-cholesterol	1 : 0.75	0	2.5	3.8	4.0	4.0	2.5
Phosphatidylcholine-cholesterol	1 : 1	0	0	0	0	1.0	2.5
Phosphatidylcholine-phosphatidyl-ethanolamine	1 : 1	0	9.0	7.0	10.0	10.5	9.0
Phosphatidylcholine-phosphatidyl-serine	1 : 1	0	5.5	7.5	14.0	15.5	15.0
Phosphatidylcholine-dipalmitoyl phosphatidylcholine	1 : 1	0	0	0	0	0	0
Phosphatidylcholine-lysolecithin	1 : 1	29.0	29.0	32.0	34.5	34.0	40.0
Phosphatidylcholine-phosphatidic acid	1 : 0.2	0	0	1.3	5.0	5.0	5.3
Phosphatidylcholine-dicetyl phosphate	1 : 1	0	9.0	9.0	10.0	11.0	9.5
Phosphatidylcholine-stearoylamine	1 : 0.2	20.0	20.0	20.0	18.5	19.0	20.0
Phosphatidylcholine-cholesterol-stearoylamine	0.7 : 0.2 : 0.1	4.0	4.0	4.0	2.0	2.0	2.0
Phosphatidylcholine-trilinolein	1 : 1	14.0	15.0	17.0	16.0	17.0	17.0
Phosphatidylcholine-tristearin	1 : 1	0	1.0	3.0	3.0	5.0	5.8

* The rate of oxidation of D-alanine by intact liposomes is expressed as percent of that by liposomes in the presence of Triton X-100. The reaction conditions are described in Materials and Methods.

perature examined. The structure of fatty acid contained also affected the incorporation of amino acid; D-alanine was incorporated more easily into phosphatidylcholine-trilinolein liposomes than into phosphatidylcholine-tristearin liposomes.

Effect of the structure of amino acids on their permeability

Since D-amino acid oxidase can oxidize various D-amino acids, the relation between the structure of amino acids and their incorporation into the liposomes was examined. The data are summarized in Table III. Among the amino acids tested, neutral amino acids were easily incorporated and the order of incorporation seems to be dependent on the hydrophobicity of carbon chain of amino acids. The order was well consistent with that reported by Klein, Moore and Smith [10], who studied the efflux of amino acids from the liposomes. Basic amino acids were scarcely incorporated into phosphatidylcholine liposomes. Among aromatic amino acids, phenylalanine and tryptophan were easily incorporated, while proline was scarcely incorporated.

Although the present data cannot be related directly to the mechanism of transfer of amino acids across the biological membranes, they seem to afford a general idea that the hydrophobic nature and the charge of permeant solute are important factors for its permeability across the membrane, besides the size of

TABLE III

EFFECT OF STRUCTURE OF AMINO ACIDS ON THEIR PERMEABILITY INTO PHOSPHATIDYLCHOLINE LIPOSOMES

D-Amino acids	Rate of oxidation *
Glycine	5.6
Alanine	7.0
Valine	14.3
Norvaline	44.6
Leucine	32.1
Isoleucine	39.3
Serine	7.1
Threonine	10.7
Proline	0.9
Phenylalanine	28.6
Tyrosine	14.3
Tryptophan	25.0
Methionine	21.4
Lysine	1.0
Arginine	1.0
Histidine	1.0

* The rate of oxidation of D-amino acid by intact liposomes is expressed as percent of that by liposomes in the presence of Triton X-100. The reaction was carried out at 30°C and the reaction system is described in Materials and Methods. The concentration of amino acid was 0.1 M in final, but a saturated solution was used when it was not dissolved to 0.1 M.

permeant solute, hydrogen bonding capacity or membrane charge noted previously [11—13].

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